GENETICS

Effects of Phosphoinositide Cycle Modifiers on Apoptosis of Peritoneal Macrophages

O. N. Aksenova, V. A. Trofimov, and T. N. Lychkina

Translated from *Byulleten' Eksperimental'noi Biologii i Meditsiny*, Vol. 137, No. 6, pp. 653-656, June, 2004
Original article submitted October 16, 2003

Hydrogen peroxide stimulates apoptosis of zymosan-activated rat peritoneal macrophages. Phosphoinositide cycle modifiers enhance the proapoptotic effect of H_2O_2 in a concentration of 1 mM and necrosis-stimulating effect of H_2O_2 in a concentration of 10 mM. Disorders in phosphoinositide metabolism and impaired reaction of the cell to proinflammatory agents are the cause of programmed cell death. Rejection of defective cells can be realized via variation of H_2O_2 concentration.

Key Words: *peritoneal macrophages; phosphoinositide cycle; hydrogen peroxide; apoptosis; necrosis*

Macrophage reactivity determines their important role in inflammatory process. Adequate reaction of macrophages under the effect of inflammation mediators depends on the efficiency of intracellular signal system functioning, primarily the phosphoinositide (PI) cycle. Second messengers formed during metabolic transformations of PI realize a wide spectrum of functional "behavioral" programs of the cell via increasing Ca^{2+} concentration in the cytoplasm. PI cycle is directly or indirectly involved in activation of proteinkinase C, calmodulin-dependent proteinkinase, tyrosine kinase, phospholipase A, calpin, Ca^{2+}/Mg^{2+}-dependent endonuclease, *etc.* [3,5,9].

The population of macrophages functioning in aggressive environment of inflammatory focus is characterized by constant renewal. Presumably, cells with impaired effective mechanisms of intracellular signaling are the first to be eliminated.

We studied the effects of PI cycle modifiers on apoptosis of peritoneal macrophages (PMP).

N. P. Ogarev Mordovian State University, Saransk. ***Address for correspondence:*** geneticlab@yandex.ru. Trofimov V. A.

MATERIALS AND METHODS

Experiments were carried out on zymosan-activated PMP of male Wistar rats (250-280 g). Zymosan (0.2 mg/g) was injected intraperitoneally 1 day before isolation of PMP. Peritoneal lavage fluid was washed in Hanks' medium on the cold and concentrated to 3×10^6/ml. PMP viability was evaluated by Trypan blue exclusion. PMP monolayers were formed on slides and cultured in Petri dishes in RPMI-1640 with 20% calf serum, 3% glutamine, penicillin (100 U/ml), and streptomycin (100 mg/ml) at 37°C for 3 h.

The mean duration of incubation with AlF_4^- (50 μM $AlCl_3$ and 10 mM NaF), phenylmethane sulfonylfluoride, LiCl, and chloropromazine (Sigma) was 40 min.

Apoptotically modified cells were detected by fluorescent microscopy using acridine orange (AO) and Hoechst 33258 (Sigma). The index of apoptosis (percentage of cells in apoptosis) was estimated. Water-soluble DNA fragments were detected using 0.2% Triton X-100, 4 mM Tris-HCl, and 1 mM EDTA as the lyzing buffer. DNA content in cell lysate (before centrifugation) and supernatant was measured by fluorometry using 20 μM Hoechst 33258 (Sigma) at

stimulation and emission wavelengths of 355 and 450 nm, respectively. The percentage of fragmented DNA was estimated as the ratio of DNA in the supernatant to total DNA in the lysate [10].

The significance of differences between the groups was evaluated using Student's *t* test.

RESULTS

There were virtually no cells with primary signs of apoptosis in the peritoneal fluid containing zymosan-activated macrophages; cell viability was 98%. Apoptosis was stimulated by H_2O_2, which activated PI cycle, tyrosine kinases, and arachidonic acid metabolism, promoted the increase in intracellular Ca^{2+} concentration by stimulating oxidative burst and phagocytosis in macrophages [1,7]. H_2O_2 (1 mM) stimulated changes in the intensity of fluorescence of AO and Hoechst 33258 incorporated into DNA of PMF (Fig. 1). The maximum fluorescence of AO shifted towards the long-wave range (from green to yellow-green). Differences in fluorescence intensity in different parts of the nucleus indicated chromatin condensation. The shape of the nucleus changed from round to irregular. Apoptotic cells accumulating Hoechst 33258 were characterized by bright green fluorescence of chromatin condensed at the periphery or were completely divided into 3-5 parts. Cells dying by necrosis were characterized by bright green fluorescence and had round swollen nucleus (Fig. 1). H_2O_2 (1 mM) stimulated the development of morphologic signs of cell death after 3 h, and apoptosis gradually increased. The apoptotic index after H_2O_2 (1 mM) stimulation was 26. Virtually no necrosis was observed in this cell population. The level of DNA fragmentation was 34%. Increasing H_2O_2 concentration to 10 mM led to an increase in the number of dead macrophages, the percentage of apoptotic cells was 30%, that of necrotically changed cells increased to 10%. AO fluorescence in necrotic cells was characterized by a shift of the radiation maximum to the red band of the spectrum. The nucleus was either blurred or looked like a swollen ball (Fig. 1). The oxidative effects of H_2O_2 are enhanced when it is used in a higher concentration, which leads to inactivation of biomolecules and subsequent cell death [6].

Hence, H_2O_2 in a concentration of 1 mM primarily triggers the apoptosis process, and in higher concen-

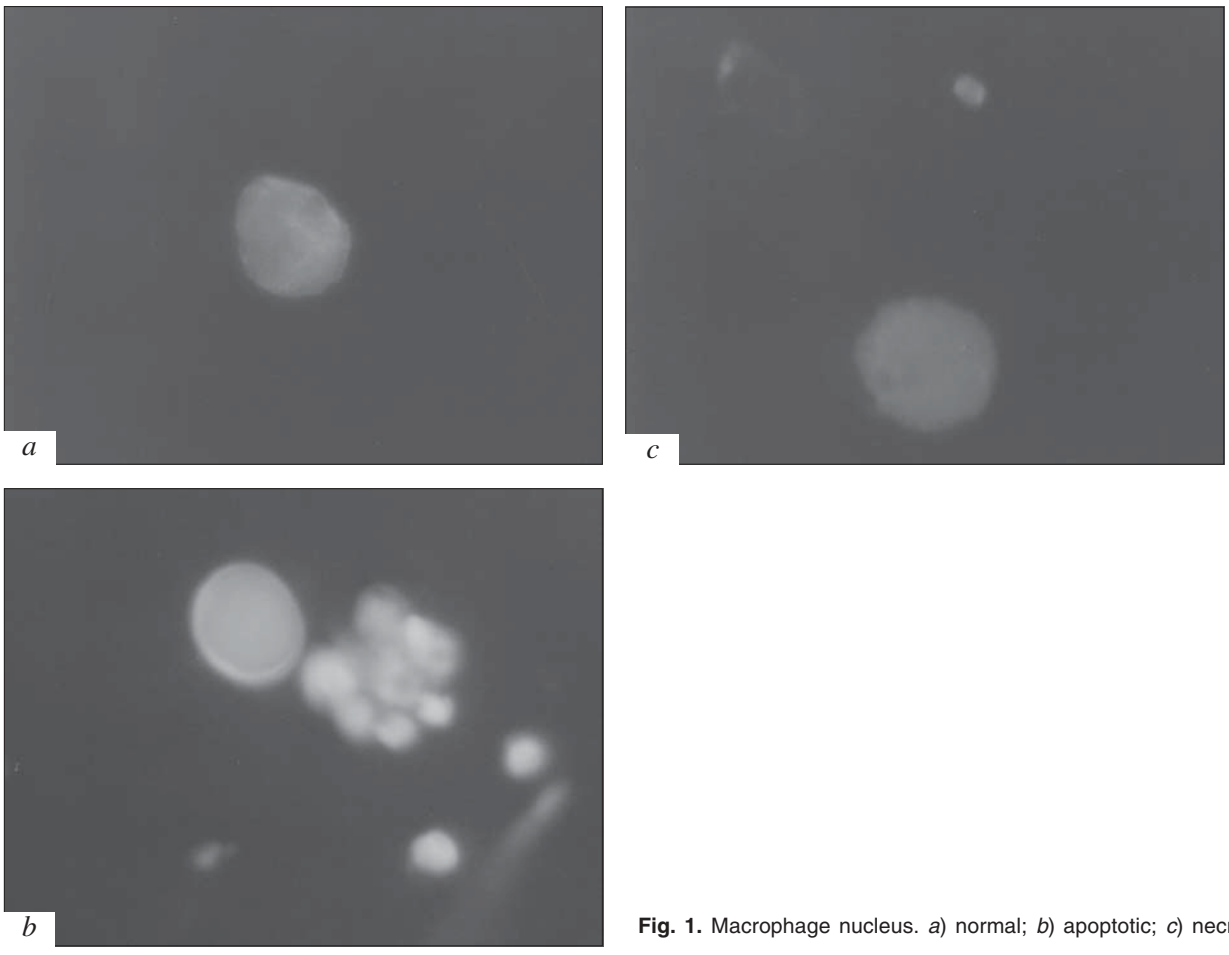

Fig. 1. Macrophage nucleus. *a*) normal; *b*) apoptotic; *c*) necrotic.

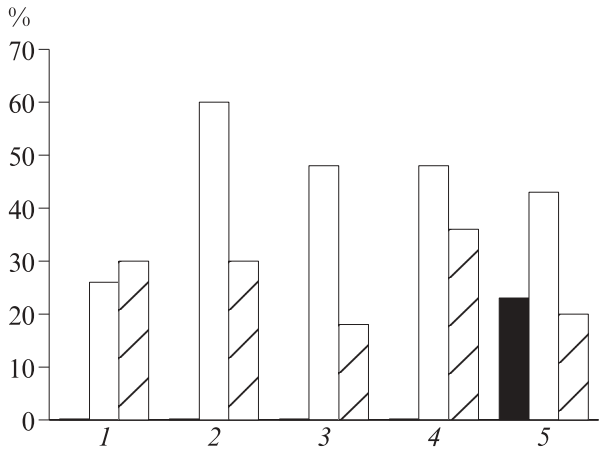

Fig. 2. Apoptotic index of peritoneal macrophages under the effects of H_2O_2 and phosphoinositide cycle modifiers. Here and in Fig. 3: *1*) H_2O_2; *2*) AlF_4; *3*) phenylmethane sulfonylfluoride; *4*) LiCl; *5*) chloropromazine. Dark bars: control; light bars: 1 mM H_2O_2; cross-hatched bars: 10 mM H_2O_2.

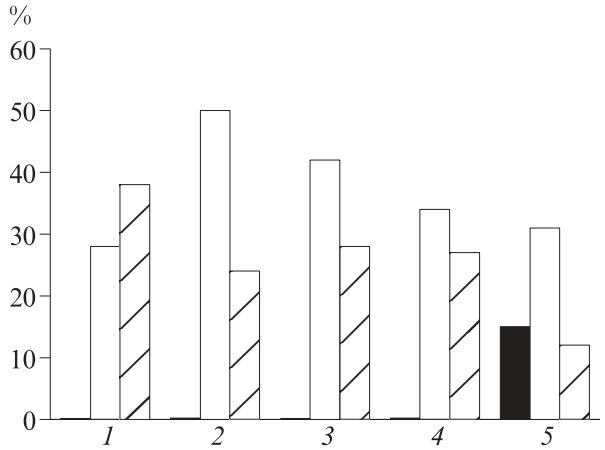

Fig. 3. Level of DNA fragmentation in rat peritoneal macrophages under the effects of H_2O_2 and phosphoinositide cycle modifiers.

trations leads to necrotic death of some macrophages. It is noteworthy that H_2O_2 can be used for rejection of defective cell.

Incubation of macrophage monolayers with AlF_4 reversibly activating heterotrimer G proteins and PI-specific phospholipase C [4] sharply enhanced the proapoptotic effect of H_2O_2 (1 mM; Fig. 2). However, the number of necrotic cells increased to 45% under the effect of H_2O_2 (10 mM) and AlF_4^-, while the apoptotic index decreased (Fig. 2). Consequently, the level of DNA fragmentation also changed (Fig. 3). Remembering all we know about the mechanisms of realization of H_2O_2 and AlF_4^- activities through PI cycle activation, let us note that their effects are eventually synergic and lead to an increase of Ca^{2+} concentration in the cytoplasm.

Phenylmethane sulfonylfluoride is an inhibitor of serine proteases and PI-specific phospholipase C [2]. Addition of H_2O_2 (1 mM) to PMP monolayers preincubated with phenylmethane sulfonylfluoride led to an increase in the number of apoptotic and necrotic cells (to 5%). Increase of effective concentration of H_2O_2 to 10 mM led to an increase in the percentage of necrotic cells (to 20%).

H_2O_2 had a similar effect on PMP preincubated with LiCl (1 mM), and inositol monophosphatase inhibitor [8] (Figs. 2, 3).

In contrast to other modifiers, chloropromazine (10 μM) exhibited a more pronounced proapoptotic effect (Figs. 2, 3) and less pronounced necrotic effect (death of ~10% cells). It inhibited metabolic transformations of PI, protein kinase C, and phospholipase A [3]. Combined exposure of cells to chloropromazine and H_2O_2 (1 mM) resulted in a lesser number of dying cells (Figs. 2, 3). Increase of H_2O_2 concentration to 10 mM led to an increase of the percentage of necrotic cells to 48.

Hence, the sensitivity of macrophages to proapoptotic effect of H_2O_2 changed under the effects of various modifiers of PI cycle. PI cycle can be activated by inflammation mediators in macrophages functioning in a focus of inflammation. PI plays an important role in the rapid response of cells to various agents. Presumably, disorders in PI metabolism and impeded formation of adequate reaction of a cell are the causes leading to apoptosis.

The study was supported by the Federal Target Program "Integration" (grant No. 3 3185).

REFERENCES

1. I. A. Gamalei and I. V. Klyubin, *Tsitologiya*, No. 12, 1233-1240 (1996).
2. R. Doson, D. Elliot, W. Elliot, and C. Jones, *Handbook of Biochemistry* [in Russian], Moscow (1991).
3. M. A. Krasil'nikov, V. M. Bezrukov, and V. A. Shatskaya, *Biokhimiya*, **57**, No. 4, 627-636 (1992).
4. Z. I. Krutetskaya and O. E. Lebedev, *Tsitologiya*, **34**, Nos. 11/12, 24-45 (1992).
5. A. A. Yarilin, *Pat. Fiziol. Eksper. Ter.*, No. 8, 38-48 (1998).
6. M. Akagi, Y. Katakuse, N. Fukuishi, *et al.*, *Biol. Pharm. Bull.*, **17**, No. 5, 732-734 (1994).
7. T. M. Battke and P. A. Sandstrom, *Immunol. Today*, **15**, No. 1, 7-10 (1994).
8. C. H. Lee, J. F. Dixon, M. Reichman, *et al.*, *Biochem. J.*, **282**, No. 2, 377-385 (1992).
9. C. J. Marshall, *Cell*, **80**, 179-185 (1995).
10. D. D. Mosser and L. H. J. Martin, *Cell. Physiol.*, **151**, 561-570 (1992).